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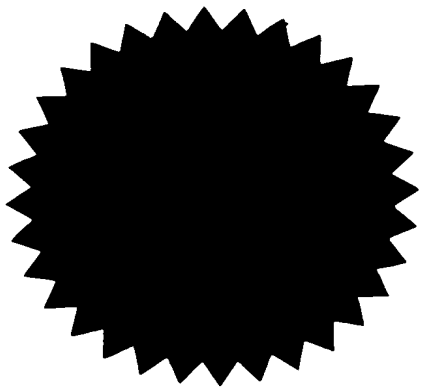

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(בעברית) שיטה לבניה במבחנה של וירוסים ופסאודווירוסים SV40
(Hebrew)

(באנגלית) In Vitro CONSTRUCTION OF SV40 VIRUSES AND PSEUDOVIRUSES
(English)

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שיטה לבניה במבחנה של וירוסים ופסאודווירוסים SV40

In Vitro CONSTRUCTION OF SV40 VIRUSES AND PSEUDOVIRUSES

Field of the Invention

The invention relates to methods for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid or exogenous protein or peptide which are particularly suitable for use in gene therapy.

Background of the Invention

Previous studies have shown that virions disrupted at pH 10.6 [Christensen, M. & Rachmeler, M. (1976) *Virology* 75:433-41] or by reducing disulfide bonds [Colomar, M.C., *et al.*, (1993) *J. Virol.* 67:2779-2786] may be reassociated to form infectious SV40 aggregates. The early attempts to package *in vitro* foreign DNA in these aggregates [Christensen & Rachmeler (1976) *ibid*] produced infectious products which did not resemble SV40 virions. Furthermore, their resistance to DNase has not been tested. Later, *in vitro* packaging experiments [Colomar *et al.* (1993) *ibid.*] did not yield particles with infectivity above the level of naked DNA.

Recently, pseudocapsids of the closely related murine polyoma virus, prepared from polyoma VP1, were used as carriers for heterologous DNA into mammalian cells [Forstova, J., *et al.* (1995) *Hum. Gene Therapy* 6:297-306]. The pseudocapsid protected 2-30% of the input DNA from DNase I digestion. When a plasmid carrying the *cat* gene was tested, most of the DNA which was protected from DNase I appeared as a ~2 kb fragment, while the input plasmid was significantly larger (exact size was not reported), suggesting that each DNA molecule was only partially protected against DNases. Infectious units were not measured in those experiments. The DNA transferred into recipient cells was functional in gene expression, albeit at a very low efficiency. With a 1.6 kb DNA fragment which carries the polyoma middle T-antigen, <30 transformed foci were obtained per 1 μg of input DNA. Similarly, a low level of CAT activity was observed with the plasmid carrying the *cat* gene. SV40 is a simian papovavirus, with a small double-stranded circular DNA genome of 5.2 kb [reviewed in Tooze, J. (1981) DNA Tumor Viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York]. The viral capsid, surrounding the viral mini-chromosome, is

composed of three viral-coded proteins, VP1, VP2, and VP3. Recent X-ray crystalographic studies on SV40 structure at 3.8Å resolution [Liddington, R., *et al.*, (1991) *Nature* **354**:278-284] revealed that the outer shell is composed of 72 pentamers of VP1, 60 hexavalent and 12 pentavalent. The pentamers have identical conformations, except for the carboxy-terminal arms, which tie them together. It appears that this construction facilitates the use of identical building blocks in the formation of a structure that is sufficiently flexible as required for the variability in packing geometry [Liddington et al., (1991) *ibid.*].

The major hindrance in beginning to use the SV40 pseudovirions in preliminary experiments in humans is the present need for a viral helper for encapsidation. This results in pseudoviral stocks that contain also wild type SV40. Because of the similarity in properties (shape, size and density) between the pseudovirions and the helper, they cannot be separated by physical means. An ideal way to prepare pseudovirions for therapeutic purposes for human use would be by *in vitro* packaging. This will provide maximal safety, since all steps of the preparation will be well controlled. *Ex vivo* administration will circumvent problems associated with immune response.

Viral packaging *in vivo* occurs by gradual addition and organization of capsid proteins around the SV40 chromatin [Garber, E.A., *et al.* (1980) *Virology* **107**:389-401]. The three capsid proteins VP1, VP2 and VP3 bind to DNA non-specifically [Soussi, T. (1986) *J. Virol.* **59**:740-742; Clever, J., *et al.* (1993) *J. Biol. Chem.* **268**:20877-20883]. How the specific recognition between the viral capsid proteins and its DNA is achieved remains unclear. The packaging of SV40 using pseudovirions, in which most of the viral DNA is replaced by other sequences has been investigated [Oppenheim, A., *et al.* (1986) *Proc. Natl. Acad. Sci. USA* **83**:6925-6929]. The pseudoviral particles are prepared by encapsidating plasmids that carry the SV40 origin of replication (*ori*) and the packaging signal (*ses*) [Oppenheim, A., *et al.* (1992) Development of somatic gene therapy: A simian virus 40 pseudoviral vector for hemopoietic cells. In *Genetic Diversity Among Jews* (Bonne-Tamir, B., A. Adam, eds), pp. 365-373, Oxford University Press, Oxford]. This

model suggests that *ses* serves several functions in SV40 packaging: as a sensor for the level of the late viral proteins in the transition from replication and/or transcription to packaging, in nucleosomal reorganization and the initiation of viral assembly [Oppenheim, A., *et al. J. Mol. Biol.* **238**:501-513] and probably as a nucleation center for viral assembly.

The pseudovirions, carrying various genes of therapeutic interest, are very efficient in DNA transfer into a wide range of cells, including human bone marrow cells, and are therefore potential vectors for gene therapy [Oppenheim *et al.* (1986) *ibid.*; Oppenheim, A., *et al.* (1987). *Ann. New. York. Acad. Sci.* **511**:418-427; Dalyot, N. & Oppenheim, A. (1989). Efficient transfer of the complete human beta-globin gene into human and mouse hemopoietic cells via SV40 pseudovirions. In: Gene Transfer and Gene Therapy (Beaudet, A.L., Mulligan R., I.M. Verma, eds), pp. 47-56, Alan R. Liss, Inc., New York; Oppenheim, A., *et al.* (1992) *J. Virol.* **66**:5320-5328]. The ideal way to prepare pseudovirions for therapeutic purposes for human use is by *in vitro* packaging. This will provide maximal safety, since all steps of the preparation will be well controlled.

Summary of the Invention

The present invention relates to a construct capable of infecting a mammalian cell comprising at least one semi-purified or pure SV40 capsid protein and exogenous DNA encoding an exogenous protein product or a vector comprising exogenous DNA encoding an exogenous protein product or exogenous RNA encoding an exogenous protein product, or an exogenous protein product, optionally further comprising operatively linked regulatory elements sufficient for the expression of said exogenous protein in a mammalian cell.

The construct of the invention preferably comprises at least one semi-purified or pure SV40 capsid protein VP1 or VP2 or VP3, or mixtures thereof.

In addition to the SV40 capsid proteins, the constructs of the invention may carry exogenous circular or linear DNA encoding an exogenous protein product, or itself a therapeutic product, or a vector comprising exogenous DNA encoding an exogenous protein product, exogenous RNA, or an exogenous protein or peptide product.

The invention also relates to method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid comprising the steps of (a) bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with said nucleic acid to give recombinant SV40 viruses or with a vector comprising said exogenous nucleic acid to give pseudoviruses; and (b) optionally subjecting the SV40 viruses or pseudoviruses formed in step (a) to digestion by nuclease to remove non-packaged DNA.

In a further aspect, the invention relates to a method for the *in vitro* construction of recombinant SV40 viruses or pseudoviruses comprising an exogenous protein or peptide comprising the steps of (a) bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with said exogenous protein to give recombinant SV40 viruses or pseudoviruses; and (b) optionally purifying the recombinant viruses or pseudoviruses obtained in step (a) from any non-packaged protein.

Additionally, the invention relates to mammalian cells infected with a construct of the invention or a construct obtained by the methods of the invention.

Furthermore, the invention relates to a method of providing a therapeutic DNA, RNA, protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of the SV40 viruses or pseudo-viruses the invention or of the infected cells of the invention.

The invention further relates to pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of the SV40

viruses or pseudo-viruses according to the invention or a therapeutically effective amount of infected cells according to the invention.

Brief Description of the Figures

Figure 1 **Self-assembly of the SV40 capsid proteins**

Nuclear extracts [Schreiber, 1989] were prepared from Sf9 cells infected with three recombinant baculovirus expressing VP1, VP2 and VP3. Samples were adsorbed onto Formvar-carbon-coated copper grids and stained with 1% phosphotungstate, pH 7.2. The samples were viewed in a Philips CM-12 electron microscope, used at a voltage of 100 kV, and photographed at a magnification of x75,000. The bar represents 100 mm.

Figure 2 **Infectivity of SV40 virions and pSO3cat pseudovirions packaged *in vitro***

Products of the *in vitro* packaging reaction were assayed for infectious units by *in situ* hybridization, following infection of CMT4 monolayers.

(a) Autoradiograms showing infections centers produced by pSO3cat DNA packaged *in vitro*, using nuclear extracts of Sf9 cells infected either with recombinant baculovirus expressing VP1 or with the 3 recombinant virus, as designated.

(b) Quantification of the results shown in Fig. 2a.

(c) In vitro packaging of SV40 DNA, using nuclear extracts of SF9 cells, uninfected or infected, as designated.

Figure 3 **Expression of the transmitted DNA molecules**

(a) CMT4 cells, infected with *in vitro* packaged pSO3cat DNA, were assayed for CAT activity 3 days post infection.

1 - No extract control

2 - mock infected control

3 - control cells "infected" with pSO3*cat* DNA only

4 - cells infected with *in vitro* packaged pSO3*cat*

5 - cells infected with twice the amount of *in vitro* packaged pSO3*cat*

6 - CAT extracted from *E coli*, as a positive control

(b) CV1 cells were mock infected, "infected" with DNA only, or with *in vitro* packaged SV40 DNA + capsid proteins, using nuclear extracts of infected Sf9 cells.

Detailed Description of the Invention

The present invention relates to constructs capable of infecting a mammalian cell, comprising at least one semi-purified or pure SV40 capsid protein and exogenous DNA encoding an exogenous protein product or a vector comprising exogenous DNA encoding an exogenous protein product or exogenous RNA encoding an exogenous protein product, or an exogenous protein product, optionally further comprising operatively linked regulatory elements sufficient for the expression or translation of said exogenous protein in said mammalian cell.

In specific embodiments, the constructs of the invention comprise at least one semi-purified or pure SV40 VP1 or VP2 or VP3 or a mixture of at least two of these capsid proteins.

In a further specific embodiment, the constructs of the invention comprise the three semi-purified or pure SV40 capsid proteins VP1, VP2 and VP3.

The constructs of the invention may comprise exogenous circular or linear DNA encoding an exogenous protein product, or itself a therapeutic product, or a vector comprising exogenous DNA encoding an exogenous protein product. Delivery into cells of linear DNA, by infecting the cells with constructs of the invention comprising such linear DNA, may be advantageous for recombination, i.e. integration into the cellular genome for stable expression.

Specifically, said DNA is DNA which encodes a therapeutic protein product or is itself a therapeutic product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein product

which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein product which is made or contained in said cell in defective form or is DNA which encodes a therapeutic protein product which is made or contained in said cell in physiologically abnormal or normal amount.

The therapeutic protein product can be any protein of interest, such as an enzyme, a receptor, a structural protein, a regulatory protein or a hormone. Of particular interest are proteins which are missing or defective in patients suffering genetic disorders. A specific example may be β-globin, missing in patients with β-thalassemia.

The constructs of the invention may optionally comprise SV40-derived *ori* DNA sequence, or other, additional SV40-derived DNA sequences. The exogenous DNA may optionally have, operatively linked thereto, additional DNA sequence encoding one or more regulatory elements sufficient for the expression of the exogenous protein encoded thereby in said mammalian cell.

In an additional aspect, the constructs of the invention can comprise exogenous RNA, said RNA being RNA which encodes a therapeutic protein product which is not made or contained in said cell, or is RNA which encodes a therapeutic protein product which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein product which is made or contained in said cell in defective form or is RNA which encodes a therapeutic protein product which is made or contained in said cell in physiologically abnormal or normal amount, and the necessary regulatory elements, including translation signal/s, sufficient for the translation of said protein product in said mammalian cell operatively linked thereto.

As in the embodiments containing DNA, the therapeutic protein product encoded by the exogenous RNA may be any protein of interest, such as an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

Packaging of RNA may be advantageous for "short term", transient gene activity. Packaging of RNA in SV40 pseudovirions, instead of, or in addition to DNA, will allow delivery of mRNA into mammalian cells. The mRNA should include mammalian translation signal, for example Kozak sequences. Such constructs will facilitate transient production of proteins, having high specific functions, *in vivo*.

The constructs of the invention will also enable the delivery of ribozyme RNA, which can be used in any application where specific RNA cleavage is desired, as an anti-AIDS agent or as an agent against other viral infections or for other therapeutical purposes.

In additional embodiments, the constructs of the invention may comprise an exogenous protein or peptide product.

In preferred such constructs, said exogenous protein or peptide product is, respectively, a therapeutic protein or peptide product which is not made or contained in said cell, or is a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is a therapeutic protein or peptide product which is made or contained in said cell in defective form or is a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount.

The delivery of packaged proteins or peptides will also facilitate their transient function *in vivo*. This approach will be used when long term effects of the packaged protein are not required or may be dangerous. Thus, for example, the delivery of packaged proteins may be useful in cases where transient local production of appropriate growth factors, for example FGF (Fibroblast Growth Factor), is required, to accelerate internal wound healing or post-operative incision healing. Local transient introduction of blood clotting factors may be desirable for prevention of hemorrhage and introduction of anti-coagulating factors may be desirable for dissolving unwanted blood clots. Application of infecting pseudovirions on site may be by catheters or any other suitable physical means.

Some proteins may have specific function on the fate of DNA delivery. The constructs of the invention will enable the delivery of mRNA encoding for a protein which promotes homologous recombination, or the delivery of such protein itself. Pseudovirions carrying a gene will be used in coinfection, together with constructs comprising mRNA coding for proteins which promote homologous recombination, such as REC A, or construct comprising such protein/s. This technique will enable gene replacement therapy.

The constructs of the invention are capable of infecting mammalian cells. Specific cells may be hemopoietic cells, such bone marrow cells, peripheral blood cells and cord blood cells, or liver cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells and germ line cells. Preferably, said mammalian cells are human cells.

In a second aspect, the invention relates to a method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid, comprising the steps of (a) bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with said nucleic acid to give recombinant SV40 viruses or with a vector comprising said exogenous nucleic acid to give pseudoviruses; and (b) optionally subjecting the SV40 viruses or pseudoviruses formed in step (a) to digestion by nuclease to remove non-packaged DNA.

Said SV40 capsid protein are preferably semi-purified or pure SV40 VP1, VP2 or VP3.

In the method of the invention, said exogenous nucleic acid is circular or linear DNA or said exogenous nucleic acid is RNA.

Said exogenous nucleic acid preferably encodes a therapeutic protein or peptide product or is itself a therapeutic product.

In specific embodiments, said DNA or RNA are DNA or RNA which encode a therapeutic protein or peptide product which is not made or contained in said cell, or which encode a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or which encode a therapeutic protein or peptide product which is made or contained in said cell in defective form or which encode a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount.

Said exogenous DNA or RNA preferably encode a therapeutic protein or peptide product which is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

In step (a) SV40-derived *ori* DNA sequence may be added and said exogenous DNA optionally has DNA sequence encoding one or more regulatory elements sufficient for the expression of said exogenous protein in said mammalian cell operatively linked thereto.

When said nucleic acid is exogenous RNA, it has to have the necessary regulatory signals, including a translation signal, sufficient for the translation of said protein product in a mammalian cell, operatively linked thereto.

In a further embodiment, the invention relates to a method for the *in vitro* construction of recombinant SV40 viruses or pseudoviruses comprising an exogenous protein or peptide comprising the steps of (a) bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with said exogenous protein to give recombinant SV40 viruses or pseudoviruses; and (b) optionally purifying the recombinant viruses or pseudoviruses obtained in step (a) from any non-packaged protein.

In this embodiment, the said exogenous protein or peptide can be, respectively, a naturally occurring or recombinant protein or peptide, a chemically modified protein or peptide, or a synthetic protein or peptide.

Said exogenous protein or peptide product are, respectively, a therapeutic protein or peptide product not made or contained in said cell, or are a therapeutic protein or peptide product made or contained in said cell in abnormally low amount, or are a therapeutic protein or peptide product made or contained in said cell in defective form or are a therapeutic protein or peptide product made or contained in said cell in physiologically abnormal or normal amount.

The method of the invention is suitable for the preparation of constructs which are capable of infecting any suitable mammalian cell. Specific cells are hemopoietic cells, such as bone marrow cells, peripheral blood cells and cord blood cells, or liver cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells and germ line cells.

In yet a further aspect, the invention relates to a mammalian cell infected with any of the constructs of the invention, or constructs obtained by any of the methods of the invention.

Still further, the invention concerns a method of providing a therapeutic DNA, RNA or protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of any of the said SV40 viruses or pseudo-viruses or a therapeutically effective amount of said infected cells.

The invention also relates to pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of the SV40 viruses or pseudo-viruses of the invention or a therapeutically effective amount of the infected cells of the invention.

The constructs of the invention are very efficient in gene transfer into a variety of cells, including human hemopoietic cells and probably also stem cells. Thus, they may be suitable for treating a wide spectrum of diseases. Plasmids carrying the desired gene and the SV40 *ori* and, optionally *ses,* are encapsidated in COS cells, optionally with helpers, as SV40 pseudovirions, and transmitted into the target cells by viral infection. The prokaryotic DNA is removed after propagation in bacteria

and before encapsidation. The constructs include only 200 bp of SV40 DNA, with cloning capacity of over 5 kb. Thus plasmids carrying over 95% human DNA are efficiently transferred into human hemopoietic cells.

The invention provides for safer and cheaper products for medical use, which may be prepared under aseptic conditions. A major advantage is that proteins are readily made in insect cells. While semi-purified proteins (nuclear extracts) are exemplified, purified proteins can be employed. Further, DNA is prepared in bacteria and can be purified before it is packaged. This ensures high purity and high quality DNA, minimizing the chance for picking spontaneous mutations and/or rearrangements. In addition, *in vivo* pseudovirions are present in a solution which also contains constituents of the cells in which they were grown. In contrast, infection by retroviral vectors prepared *in vivo* is done by co-culturing of the patients cells with the producer cell-lines (usually murine). Although *in vivo* prepared viral vectors (such as adenovirus or adeno-associated virus) can be purified, this may substantially increase production cost, as purification is also associated with loss of virion particles. In addition, in helper-free packaging cell-lines (of any virus) there is always a risk of contamination by recombinants. These could be either the wild-type virus or unknown recombination products, carrying potentially harmful (cellular) genes. This risk is completely abolished when packaging is done *in vitro*. Moreover, *in vitro* packaging can accommodate larger plasmids than *in vivo* packaged SV40 pseudovirions: The *in vivo* packaged pseudovirions accommodate only up to ~5.4 kb of DNA [Oppenheim, A. & Peleg, A. (1989) *Gene* 77:79-86]. In the present *in vitro* method ~7.5 kb have been packaged successfully, and larger plasmids can be packaged. Regulatory elements (e.g. β-globin LCR), which interfere with packaging *in vivo*, are not expected to interfere with packaging *in vitro*. An additional important advantage is that the *ses* element is not required, reducing the size of the required SV40 sequences to ~100 bp, comprising the *ori,* in the exemplified experiments. Embodiments without even this element are also contemplated. In the present examples, the *ori* element was required for the assay of infectious units. The high flexibility afforded by the

method and constructs of the invention may allow the development of gene targeting (or gene replacement therapy).

EXAMPLES

Cloning the genes of the SV40 capsid proteins for expression in bacteria

First, plasmids designed to express the complete VP1, VP2 and VP3 polypeptides as fusion proteins to glutathion-S-transferase (GST) in *E. coli* were constructed [Smith, D. B. & Johnson, K. S. (1988). *Gene* 67:31-40]. The respective SV40 fragments were cloned into the vector with the aid of PCR. Expression level was high, leading to the production of insoluble inclusion bodies. Similarly, it was recently reported [Clever *et al.*, *ibid.*] that a truncated VP2 fused to GST also yielded an insoluble product.

Preparation of antibodies

The three GST-fusion capsid proteins were used to raise polyclonal antibodies in rabbits. Antibodies against GST-VP1 did not cross-react with VP2 and VP3. As expected, antibodies against GST-VP2 reacted both with VP2 and VP3, and did not cross-react with VP1.

Cloning the SV40 late genes for expression in insect cells

SV40 DNA fragments were cloned into the plasmid vectors pVL1393 and pVL1392 (commercially available from PharMingen, San Diego, California), derived from *Autographa californica* nuclear polyhedrosis virus (AcMNPV) [Luckow, V.A. & Summers, M.D. (1988). **6**:47-55; Luckow, V. A. & Summers, M.D. (1989). *Virology* **170**:31-39]. In these vectors expression of the foreign gene is driven by the strong promoter for the viral occlusion protein, polyhedrin. The genes for the capsid proteins were cloned into pVL1393 as follows: VP1 was cloned by introducing a *Stu*I-*Bcl*I DNA fragment (SV40 coordinates 1464-2770) into the plasmid cleaved by restriction endonucleases *Sma*I and *Bgl*II. The VP2 gene was cloned by ligating a *Hin*cII-*Eco*RI fragment (522-1782) between the *Sma*I and *Eco*RI sites. The VP3 gene, which is nested in the VP2 gene (it is translated from an internal AUG signal), was cloned by using a *Sau*3AI-*Eco*RI fragment (874-1782) and the *Bam*HI

and *Eco*RI sites of pVL1393. A fourth late polypeptide, the agnoprotein (or LP1), encoded by the leader region of the late 16S mRNA, appears to play a role in expediting virion assembly Carswell, S. & Alwine, J.C. (1986) *J Virol* **60**:1055-61; Resnick, J. & Shenk, T. (1986) *J. Virol.* **60**:1098-106]. The agnogene, a *Pvu*II-*Mbo*I fragment (273-873), was cloned between the *Sma*I and *Bam*HI sites of plasmid pVL1392.

The structures of the four recombinant plasmids were confirmed by restriction analysis and after propagation in *E. coli*. Sequence analysis was performed for The recombinant plasmids carrying VP2 and VP3. Recombinant baculovirus carrying the four respective genes were produced using the BaculoGold kit (kindly provided by PharMingen, California). The technique relies on homologous recombination between the plasmid and a modified type of baculovirus with a lethal deletion. Each of the recombinant plasmids was cotransfected, together with linearized DNA of the defective baculovirus, into *Spodoptera frugiperda* (Sf9) cells. Virus was harvested 4 days later, according to the protocol supplied by the manufacturer. To obtain high titer stocks each of the recombinant virus was amplified by 3 cycles of infection (5 cycles for the VP2 recombinant virus) of freshly seeded Sf9 cells [Summers, M. D. & Smith, G. E. (1988) A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station, College Station, Texas]. The final titers of the 4 recombinant baculovirus stocks were 2-4x10^8 pfu/ml.

The SV40 proteins were produced in Sf9 cells, infected at a multiplicity of 10 pfu/cell and grown at 27°C. Cells were harvested and the soluble proteins were analyzed by SDS-PAGE [Laemmli, E. K. (1970) *Nature* **277**: 80-685. and western blotting [Harlow, E. & Lane, D. (1988). Antibodies, a laboratory manual. Cold Spring Harbor Laboratory, N.Y, Cold Spring Harbor] using antibodies raised against the corresponding GST-fusion proteins. Kinetic studies showed increasing levels of the SV40 proteins from 3 to 6 days postinfection.

The three capsid proteins assemble spontaneously to form SV40-like particles

The SV40 capsid proteins were produced, separately, in *Spodoptera frugiperda* (SF9) cells from recombinant baculovirus each carrying the genes coding for VP1, VP2 or VP3. The cells were harvested and nuclear and cytoplasmic fractions were analyzed by SDS-PAGE and western blotting. The results demonstrated that VP1 and VP3 were preferentially present in the nuclear fraction, whereas VP2 was preferentially cytoplasmic (not shown). When the three proteins were co-expressed in the same cells (following infection with the three recombinant baculovirus together), all three proteins were present in the nuclear fraction. The capsid proteins self-assembled to form SV40-like structures of various sizes (Fig. 1).

To purify VP1, nuclear extracts of infected cells were placed on 15-35% glycerol gradient with a 2M sucrose cushion. Western blot analysis and EM studies demonstrated that the majority of VP1 was present as high MW structures in 2 peaks, one at the cushion and the other at the bottom of the tube. About a third of the protein was present as pentamers (10 S units; MW ~210kd). In most of the experiments, monomers were not seen. The results indicate that VP1 molecules readily form pentamers and high MW structures at this high concentration. VP1 pentamers and higher MW structures are stabilized by S-S bonds [Gharakhanian, E., *et al.* (1995) *Virology* **207**:251-254] and by Ca^{++} [Liddington *et al.*, *ibid.*]. It appears that only at very low concentrations VP1 molecules may remain monomeric (Gharakhanian *et al.*, *ibid.*].

Similar experiments, performed with nuclear extracts of cells containing the 3 capsid proteins, demonstrated that the three proteins co-precipitated together in the glycerol gradients in two high MW fractions, at the very bottom of the tube (fraction I) and at the cushion (fraction II). In addition, peaks corresponding to VP1 pentamers and to VP2 monomers were also seen.

In SV40, the 3 capsid proteins are expressed from a single promoter with complex regulatory controls, including several transcription start sites,

alternative splicing to two major species, 16S RNA, producing the agnoprotein (which is not part of the capsid) and VP1 and 19S, producing VP2 and VP3. The two bicistronic messages contain internal translation initiation signals. This organization is thought to facilitate coordinated expression at the correct ratio for packaging [Sedman, S. A., *et al.* (1990) *J. Virol.* **64**:453-457; Sedman, S. A., *et al.* (1989). *J. Virol.* **63**:3884-3893]. Co-production of the capsid proteins VP1, VP2 and VP3 in SF9 cells was performed by infecting with the 3 baculovirus species at equal multiplicities. Nevertheless, the ratio of the 3 proteins in fraction II was similar to the ratio obtained in monkey CMT4 cells [Gerard, R.D. & Gluzman, Y. (1985) *Mol. Cell. Biol.* **5**:3231-3240] infected with wild type SV40. This was not true for fraction I. Furthermore, EM studies revealed that fraction II was relatively homogeneous, containing particles of approximately SV40 size. On the other hand the particles in fraction I were highly heterogeneous in size.

DNA is packaged in the SV40 capsids in vitro, forming infectious particles

Packaging experiments were performed with SV40 DNA and with heterologous plasmid DNA. The capsid proteins were mixed with 1 μg of SV40 or pSO3*cat* [Oppenheim *et al.* (1986) *ibid.*] DNA in a total volume of 4 μl, and incubated at 37° for 6 hrs to interact with the DNA. The mixture was then transferred to ice and incubated for 1 hr after the addition of Ca^{++} and Mg^{++} to final concentrations of 0.1 mM and 8 mM, respectively. This was followed by DNase I treatment, to remove DNA which was not stably packaged. The mixture was added to CMT4 monolayers using a standard SV40 infection protocol. CMT4 are monkey kidney cells which harbor the gene for the SV40 T-antigen under control of the metallothionein promoter [Gerard & Gluzman *ibid.*]. Low level constitutive T-antigen production, without induction, was sufficient for plasmid DNA replication in these experiments. The cells were incubated for approximately 40-60 hrs, to allow viral DNA replication in the infected cells. The monolayers were then transferred onto nitrocellulose membranes and assayed for infectious centers by *in situ* hybridization (Fig. 2a). This procedure yielded infectious units both with SV40 DNA (Fig. 2c) and with pSO3*cat* DNA (Fig. 2a,b). A typical experiment,

demonstrating infectivity of *in vitro* packaged pSO3*cat*, is shown in Fig. 2a. Under these conditions naked DNA sometimes also entered the cells. However, the DNase I treatment completely removed the naked DNA background from the assay. The experiments showed that using the nuclear extracts containing VP1+VP2+VP3 directly, without purification on glycerol gradients, yielded better results both for SV40 DNA and for pSO3*cat* DNA (not shown).

Insect cells were infected with 3 recombinant baculovirus encoding for the three capsid proteins, VP1, VP2 and VP3, at moi 10 each. After 4 days nuclear extracts were prepared essentially as described by Schreiber [Schreiber, E., *et al.* (1989) *Nucleic Acids Res.* **15**:6419-6436] by shaking the nuclei, isolated with 10% NP-40, in a buffer containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF and 1 mM leupeptin. DTT, PMSF and leupeptin were added just before use. The nuclei from each 75 cm^2 culture bottle were extracted with 150 µl buffer.

The nuclear extract was used in packaging experiments performed exactly as described above. For each packaging reaction 2 µl nuclear extracts were mixed with 1µl DNA (either SV40 or pSO3*cat*) in a total volume of 4 µl, for 6 hrs at 37°C. The reaction mixture was transferred to ice, 2 µl of 0.4 mM $CaCl_2$/24 mM $MgCl_2$ were added and incubated for 60 min. on ice. This was followed by the addition of 2 µl containing 0.5 unit of DNase I, and the incubation continued for 10 min. on ice. The reaction was stopped by the addition of 2µl of 25 mM EDTA (final concentration 5 mM) Serum-free medium was added (300 µl) and the mixture was applied to a sub-confluent CMT4 culture in a 6 cm diameter culture plate. The cultures were incubated at 37°C with gentle agitiation every 20 min. Two hours later the infection mixture was sucked off and 5 ml fresh medium containing 5% FBS was added. After ~40 hrs, to allow replication of the DNA transmitted by the infectious particles, the monolayers were transferred to nitrocellulose layers and processed for hybridization.

The experiments showed that VP1 alone is capable of producing infectious particles (Fig. 2a,b,c). However, the addition of DNase I to the packaging mixture before infection reduced the number of infectious units produced with VP1 alone. This was seen both for pSO3*cat* DNA (Fig. 2b) and for SV40 DNA (Fig. 2c), suggesting that the DNA in those particles was not as effectively protected from DNase I, under the conditions described above. The results suggest that VP2 and VP3 contribute to the stability of the particles. Fig. 2c also demonstrates that presence of the SV40 capsid proteins is required for the production of infectious particles, as "packaging" with nuclear extracts of uninfected Sf9 gave negative results. Few "infectious units" were seen in cells treated with SV40 DNA only, demonstrating the ability of naked DNA to penetrate cells. DNase I treatment completely removed this background.

The infectious particles transmit complete DNA molecules which are biologically functional

The results shown in Fig. 2c may presumably reflect DNA which did not penetrate the cells but remained adsorbed on the cell surface, in structures which are DNase I resistant. To prove that the *in vitro* packaged DNA entered the cells, and that the DNA is biologically active in gene expression, we asked whether the *cat* gene transmitted by pSO3*cat* expressed the CAT enzyme. CMT4 cells were harvested 3 days postinfection with *in vitro* packaged pSO3*cat*. CAT assays [Oppenheim *et al.* (1986) *ibid.*] demonstrated enzyme activity at a significant level (Fig. 3a), indicating that the infectious units transmitted biologically active DNA into the cells.

It was then to be verified whether complete DNA molecules became packaged in this experimental protocol. For production of SV40 virions, the complete SV40 molecule is required, including the regulatory region, and the early and the late genes. SV40 DNA was packaged as described above and used to infect CV-1 cells. After 2 weeks extensive cell lysis was visible, indicating that productive SV40 infection was going on (Fig. 3b). Only complete SV40 DNA, which can produce functional T-antigen as well as the late proteins, can produce virions on CV-1 cells. It was

therefore concluded that the *in vitro* packaging system described here produces particles which contain the complete circular DNA molecules.

Plasmids significantly larger than SV40 can be packaged in vitro

Further packaging experiments were performed under the same conditions as before, except that instead of SV40 or pSO3*cat* DNA other plasmids were used. In each experiment, 1 μg of plasmid DNA was incubated with 2 μl of nuclear extracts of Sf9 cells infected with VP1+VP2+VP3. The experiments were repeated at least twice for each plasmid. Typical results are shown in Table 1.

Table 1
In Vitro Packaging of Various Plasmid

Plasmid	Properties	Size (kb)	Infectious Units
SV40		5.2	~250
pSO3*cat*		4.1	142
pSO6β-1	carries β-globin	7.3	~200
pSO6β-9	carries β-globin + LCR element	7.0	56
pSM1	carries MDR1	7.1	44

Importantly, the experiments demonstrate that various plasmids, carrying useful genes, can be packaged. Furthermore, packaging *in vitro* is not limited to 5.3-5.5 kb of plasmid DNA, and plasmids over 7 kb can also be packaged. This is presumably because under *in vitro* conditions naked DNA is packaged, rather than a minichromosome (which includes the DNA complexed in nucleosomes). The minichromosome occupies much more space, as compared to DNA of the same size, within the pseudoviral particle.

As seen in Fig. 1, occasionally 2 or 3 virion-like particles are joined together. These aggregates may allow packaging of DNA of unlimited size.

In vitro packaging is probably accomplished by a mechanism which is different from the packaging in vivo. In the latter process, the viral capsid proteins are thought to assemble around the viral minichromosome, while *in vitro*, empty capsid-like structure (Fig. 1) serve as starting material. Furthermore, *in vitro* packaging utilizes naked DNA, prepared in *E. coli*. This result, combined with many of the inventors' previous studies on *in vivo* packaging, leads to the prediction that potent regulatory squences, such as the β-globin LCR, which interfere with viral packaging *in vivo* [Chang *et al.* (1992) *ibid.*; Dalyot (1994) *ibid.*], will not interfere with *in vitro* packaging. This will allow to combine in the constructs for gene therapy the optimal regulatory signal, which will lead to important improvement in expression of the delivered genes.

Claims

1. A construct capable of infecting a mammalian cell comprising at least one semi-purified or pure SV40 capsid protein and exogenous DNA encoding an exogenous protein product or a vector comprising exogenous DNA encoding an exogenous protein product or exogenous RNA encoding an exogenous protein product, or an exogenous protein product, optionally further comprising operatively linked regulatory elements sufficient for the expression of said exogenous protein in a mammalian cell.

2. A construct according to claim 1 comprising a mixture of at least two semi-purifies or pure SV40 capsid proteins.

3. A construct according to claim 1 or claim 2 comprising a mixture of three semi-purified or pure SV40 capsid proteins.

4. A construct according to any one of claims 1 to 3 wherein said SV40 capsid protein is semi-purified or pure VP1 or VP2 or VP3.

5. A construct according to any one of claims 1 to 4 comprising exogenous circular or linear DNA encoding an exogenous protein product, or itself a therapeutic product, or a vector comprising exogenous DNA encoding an exogenous protein product.

6. A construct according to claim 5 wherein said DNA is DNA which encodes a therapeutic protein product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein product which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein product which is made or contained in said cell in defective form or is DNA which encodes a therapeutic protein product which is made or contained in said cell in physiologically abnormal or normal amount.

7. A construct according to claim 6 wherein said therapeutic protein product is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

8. A construct according to any one of claims 5 to 7 comprising SV40-derived *ori* DNA sequence and comprising a DNA sequence encoding one or more regulatory elements sufficient for the expression of said exogenous protein in said mammalian cell.

9. A construct according to any one of claims 1 to 4 comprising exogenous RNA, wherein said RNA is RNA which encodes a therapeutic protein product which is not made or contained in said cell, or is RNA which encodes a therapeutic protein product which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein product which is made or contained in said cell in defective form or is RNA which encodes a therapeutic protein product which is made or contained in said cell in physiologically abnormal or normal amount, said RNA having regulatory elements, including translation signal, sufficient for the translation of said protein product in said mammalian cell, operatively linked thereto.

10. A construct according to claim 9 wherein said therapeutic protein product is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

11. A construct according to any one of claims 1 to 4 comprising an exogenous protein or peptide product.

12. A construct according to claim 11 wherein said protein or peptide product is, respectively, a therapeutic protein or peptide product which is not made or contained in said cell, or is a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is a therapeutic protein or peptide product which is made or contained in said cell in defective form or is a therapeutic protein or peptide product which is made or

contained in said cell in physiologically abnormal or normal amount.

13. A construct according to any one of the preceding claims wherein said cell is selected from the group consisting of hemopoietic cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells and germ line cells.

14. A construct according to claim 13 wherein said hemopoietic cells are bone marrow cells, peripheral blood cells and cord blood cells, or liver cells.

15. A method for the *in vitro* construction of SV40 viruses or pseudoviruses comprising exogenous nucleic acid comprising the following steps:

(a) bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with said nucleic acid to give recombinant SV40 viruses or with a vector comprising said exogenous nucleic acid to give pseudoviruses; and

(b) optionally subjecting the SV40 viruses or pseudoviruses formed in step (a) to digestion by nuclease to remove non-packaged DNA.

16. A method according to claim 15 wherein said SV40 capsid protein is semi-purified or pure SV40 VP1, VP2 or VP3.

17. A method according to claim 15 or claim 16 wherein said exogenous nucleic acid is circular or linear DNA.

18. A method according to claim 15 or claim 16 wherein said exogenous nucleic acid is RNA.

19. A method according to claim 15 wherein said exogenous nucleic acid encodes a therapeutic protein product or is itself a therapeutic product.

20. A method according to any one of claim 17 or claim 19 wherein said DNA is DNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is DNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount.

21. A method according to claim 20 wherein said exogenous DNA encodes a therapeutic protein or peptide product which is an enzyme, a receptor, a structural protein, a regulatory protein or a hormone.

22. A method according to claim 15 wherein in step (a) SV40-derived *ori* DNA sequence is added and said exogenous DNA optionally has DNA sequence encoding one or more regulatory elements sufficient for the expression of said exogenous protein in said mammalian cell operatively linked thereto.

23. A method according to any one of claims 15 wherein said nucleic acid is exogenous RNA, wherein said RNA is RNA which encodes a therapeutic protein or peptide product which is not made or contained in said cell, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in abnormally low amount, or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in defective form or is RNA which encodes a therapeutic protein or peptide product which is made or contained in said cell in physiologically abnormal or normal amount and wherein said RNA has regulatory elements, including translation signal, sufficient for the translation of said protein product in said mammalian cell, operatively linked thereto.

24. A method for the *in vitro* construction of recombinant SV40 viruses or pseudoviruses comprising an exogenous protein or peptide comprising the following steps:
    (a) bringing a semi-purified or purified SV40 capsid protein or a mixture of at least two such proteins into contact with said exogenous protein to give recombinant SV40 viruses or pseudoviruses; and
    (b) optionally purifying the recombinant viruses or pseudoviruses obtained in step (a) from any non-packaged protein.

25. A method according to claim 24 wherein said exogenous protein or peptide are, respectively, a naturally occurring or recombinant protein or peptide, a chemically modified protein or peptide, or a synthetic protein or peptide.

26. A method according to claim 26 wherein said exogenous protein or peptide product are, respectively, a therapeutic protein or peptide product not made or contained in said cell, or are a therapeutic protein or peptide product made or contained in said cell in abnormally low amount, or are a therapeutic protein or peptide product made or contained in said cell in defective form or are a therapeutic protein or peptide product made or contained in said cell in physiologically abnormal or normal amount.

27. A method according to any one of claims 15 to 26 wherein said cell is selected from the group consisting of hemopoietic cells, epithelial cells, endothelial cells, liver cells, epidermal cells, muscle cells, tumor cells and germ line cells.

28. A method according to claim 27 wherein said hemopoietic cells are bone marrow cells, peripheral blood cells and cord blood cells, or liver cells.

29. A mammalian cell infected with a construct of any one of claims 1 to 14.

30. A mammalian cell infected with a construct obtained by the method of any one of claim 15 to 28.

31. A method of providing a therapeutic DNA, RNA, protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of the SV40 viruses or pseudoviruses according to any one of claims 1 to 14.

32. A method of providing a therapeutic DNA, RNA, protein or peptide product to a patient in need of such product by administering to said patient a therapeutically effective amount of infected cells according to any one of claims 20 or 30.

33. Pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of the SV40 viruses or pseudo-viruses according to any one of claims 1 to 14.

34. Pharmaceutical compositions comprising as active ingredient a therapeutically effective amount of infected cells according to any one of claims 29 or 30.

A. E. MULFORD
ATTORNEYS FOR APPLICANTS

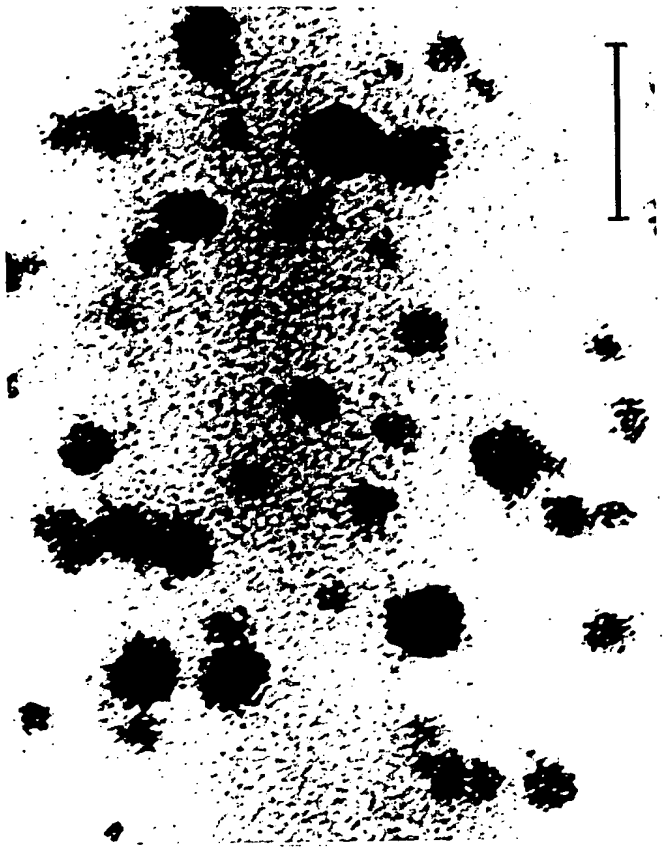


Fig. 1

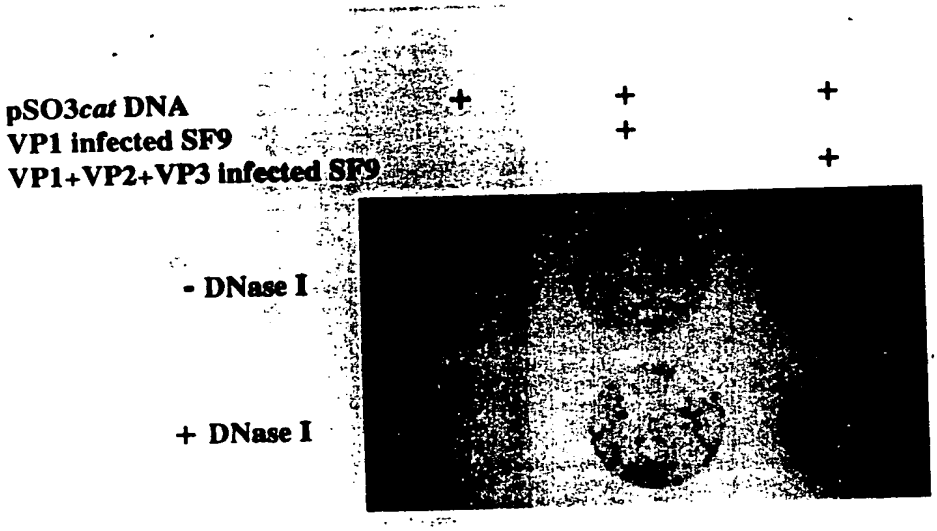

pSO3cat DNA
VP1 infected SF9
VP1+VP2+VP3 infected SF9
+
+
+
+
+
- DNase I
+ DNase I

Fig. 2a

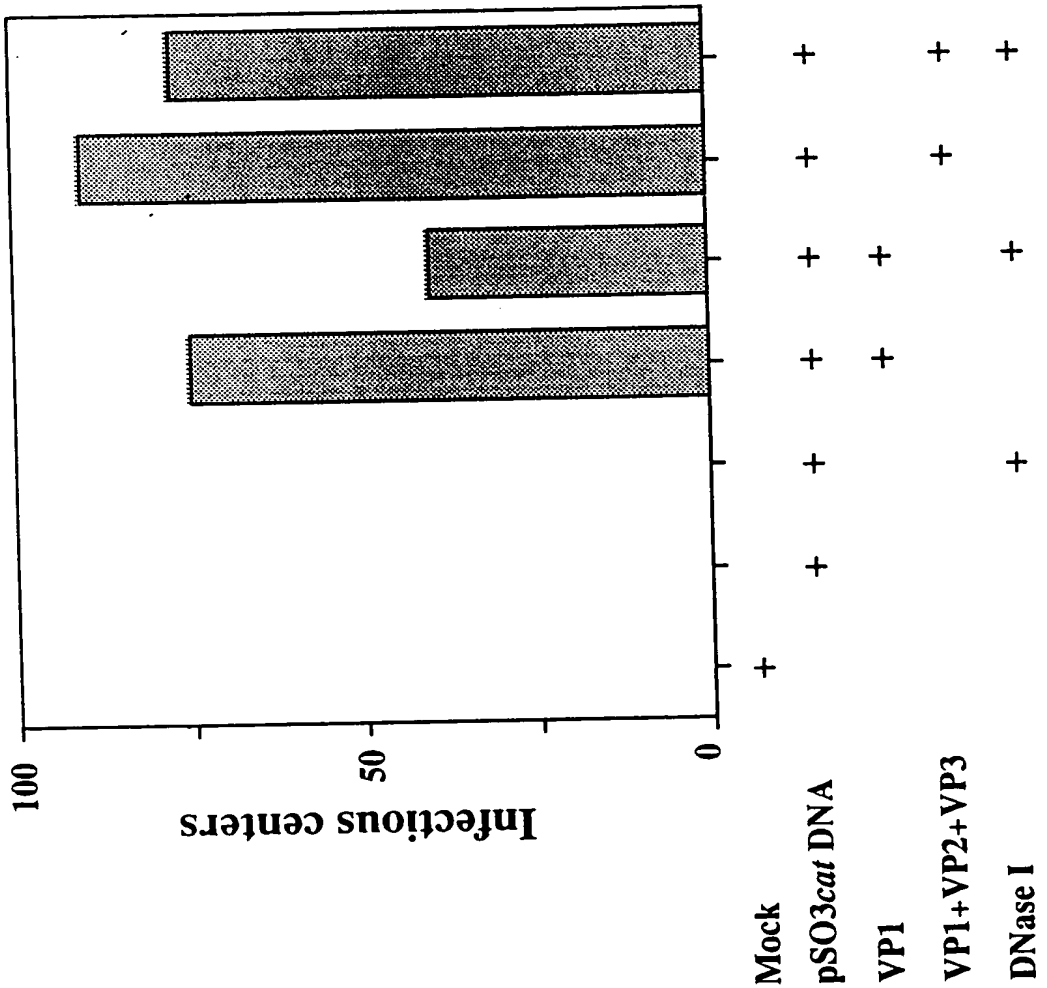

Infectious centers
100
50
0
Mock
pSO3cat DNA
VP1
VP1+VP2+VP3
DNase I

Fig. 2b

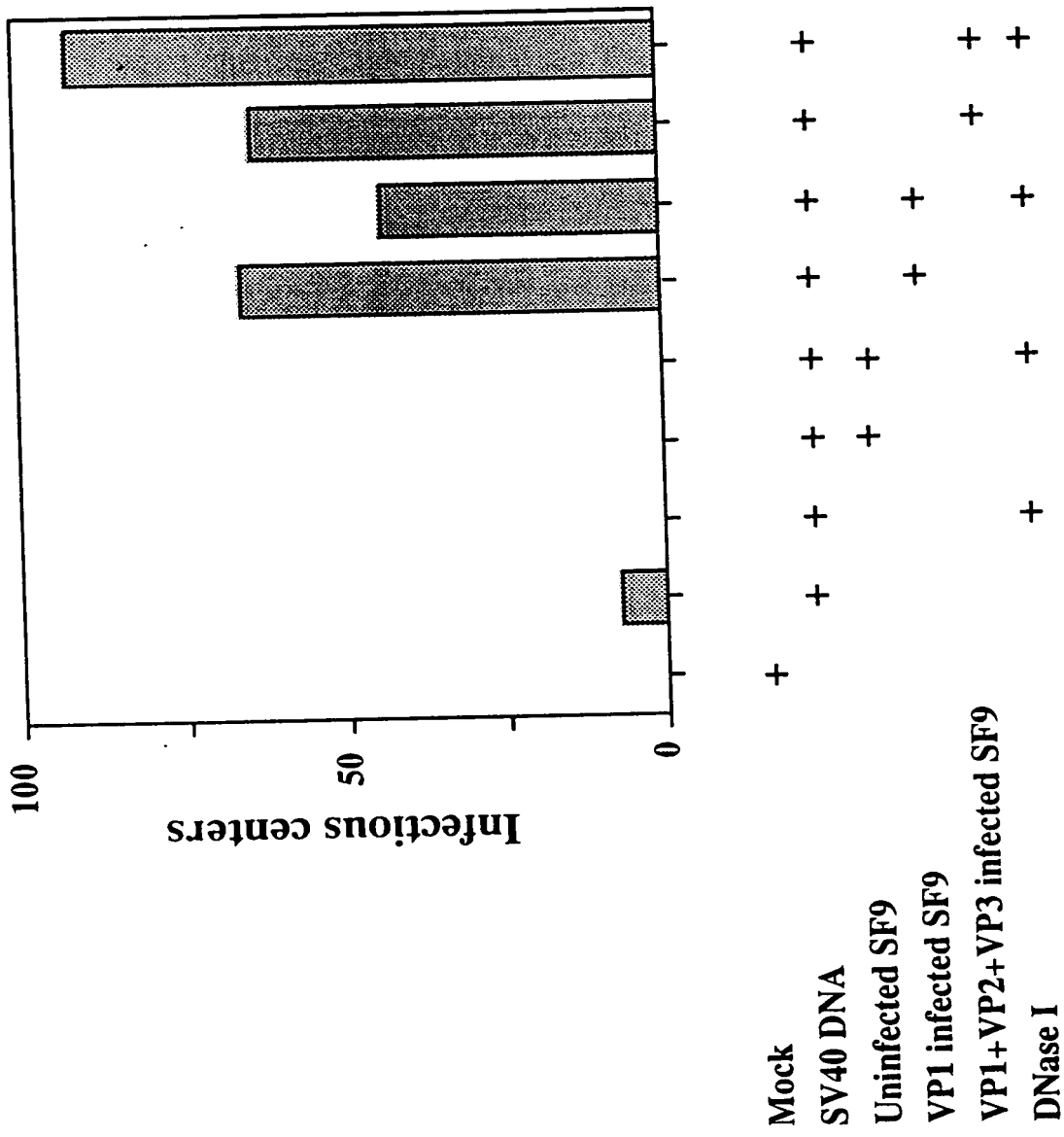

Infectious centers
100
50
0
Mock
SV40 DNA
Uninfected SF9
VP1 infected SF9
VP1+VP2+VP3 infected SF9
DNase I

Fig. 2c

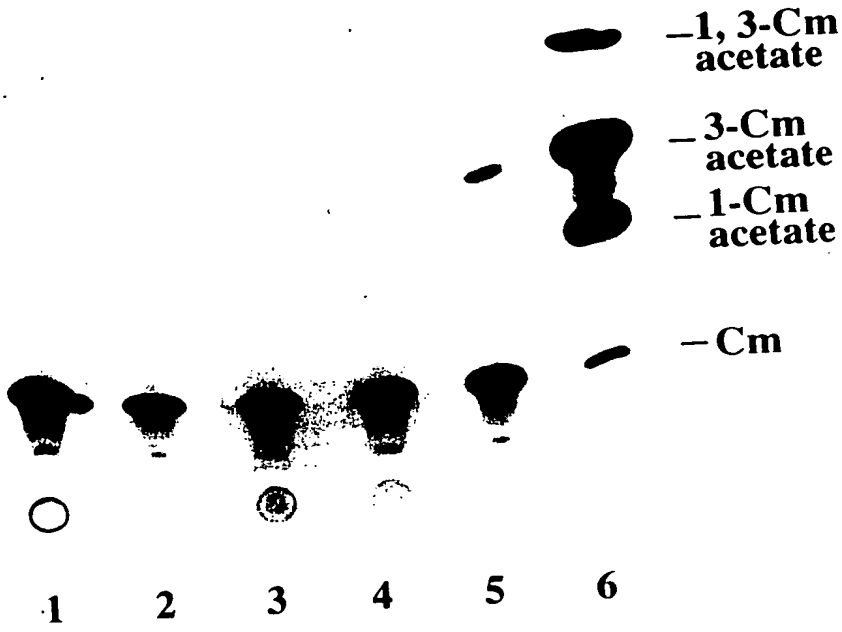
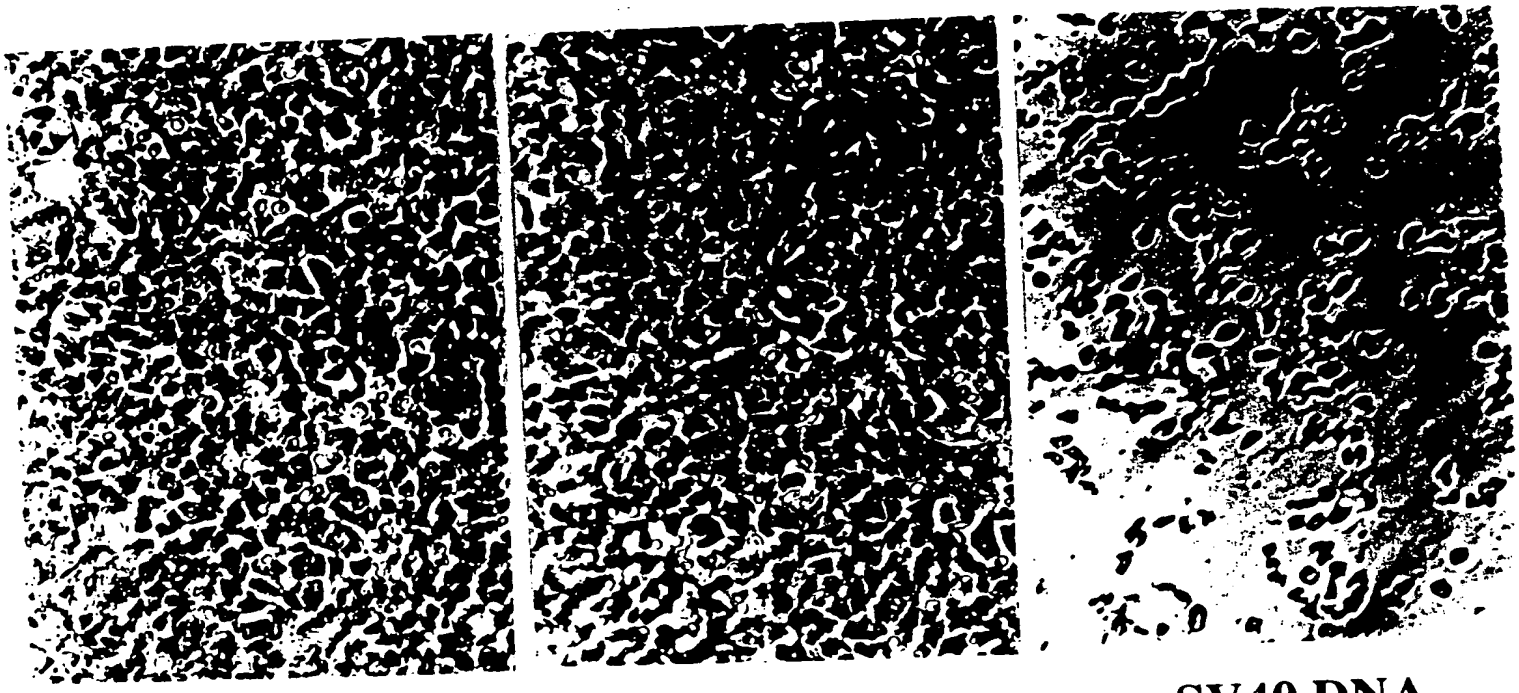


Fig. 3a



Mock

SV40 DNA

SV40 DNA
+ capsid proteins

Fig. 3b